APPENDIX A

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Monoclonal Antibodies to CNA, a Collagen-binding Microbial Surface Component Recognizing Adhesive Matrix Molecules, Detach *Staphylococcus aureus* from a Collagen Substrate*

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Previous studies showed that *Staphylococcus aureus* expresses a collagen-binding MSCRAMM (Microbial Surface Component Recognizing Adhesive Matrix Molecules), CNA, that is necessary and sufficient for *S. aureus* cells to adhere to cartilage and is a virulence factor in experimental septic arthritis. We have now used a monoclonal antibody (mAb) approach to further analyze the structure and function of CNA. 22 mAbs raised against the minimal ligand binding domain, CNA-(151–318), were shown to bind to the MSCRAMM with similar affinity. All mAbs appear to recognize conformation-dependent epitopes that were mapped throughout the CNA-(151–318) domain using a chimeric strategy where segments of CNA are grafted on ACE, a structurally related MSCRAMM from *Enterococcus faecalis*. These mAbs were able to inhibit ^{125}I-collagen binding to CNA-(151–318) as well as to intact *S. aureus* cells. They also interfered with the attachment of bacteria to collagen substrates. Furthermore, some of the mAbs could effectively displace ^{125}I-collagen bound to the bacteria. These displacing mAbs were also able to detach bacteria that had adhered to a collagen substrate in a preincubation, raising the possibility that some of the mAbs may be used as therapeutic agents.

Staphylococcus aureus is a multipotential bacterial pathogen that can colonize a wide range of host tissues and cause a variety of infections such as wound infections, endocarditis, pneumonia, osteomyelitis, and septic arthritis. As with most infectious agents, initial attachment of *S. aureus* to the host tissues is considered the first crucial step in the disease process. Staphylococci produce a family of adhesins of the MSCRAMM (Microbial Surface Component Recognizing Adhesive Matrix Molecules)[1] type that mediate adherence to extracellular matrix (ECM) proteins. Collagen is the main component of the ECM of most tissues, and *S. aureus* can express a collagen-binding MSCRAMM, called CNA. Switalski *et al.* (1) demonstrated that CNA was necessary and sufficient for *S. aureus* to attach to cartilage *in vitro*, suggesting that CNA can act as an adhesin. Furthermore, CNA was shown to be a virulence factor in experimental septic arthritis (2). CNA$^+$ strains showed substantially increased virulence compared with the isogenic CNA$^-$ strains as demonstrated by macroscopic clinical evaluation and histopathological analysis of the joints. No viable *S. aureus* cells were recovered from the joints of mice injected with the CNA$^-$ strains, although significant numbers of *S. aureus* cells were isolated from the joints of those injected with the CNA$^+$ strains. In addition, vaccination with a recombinant fragment of CNA protected mice from septic death induced by intravenously administered *S. aureus* (3).

Other bacteria can adhere to collagen substrates or bind soluble collagen, including some strains of *Escherichia coli* (4), *Yersinia enterocolitica* (5–7), *Klebsiella pneumoniae* (8), *Streptococcus mutans* (9, 10), group A streptococci (11, 12), *Streptococcus gordonii* (10, 13), *Enterococcus faecalis* (14, 15), and *Lactobacillus reuteri* (16). A collagen adhesin YadA was identified in *Y. enterocolitica* and shown to contribute to the arthritogenicity of the bacteria in a rat model (17). Substitution of two histidine residues in YadA with alanine abrogated bacterial binding and adherence to collagen, and reduced the virulence of the bacteria in a mouse model (18), suggesting that collagen binding is an important step in the *Y. enterocolitica* disease process.

CNA has structural features characteristic of cell wall-anchored proteins on Gram-positive bacteria. CNA consists of an N-terminal signal peptide, a non-repetitive A region, one to four repeated units (B-region), followed by a cell wall anchor region, a transmembrane segment, and a short positively charged cytoplasmic tail. The A region of CNA-(30–531) was found to be fully responsible for the collagen binding activity of CNA (19, 20). The minimum binding domain was localized to a 19-kDa fragment, CNA-(151–318) (formerly designated M19- and CBD-(151–318)), within CNA-(30–529) (19). Recombinant CNA-(151–318) was crystallized and its structure solved (21). CNA-(151–318) forms a β-barrel composed of two antiparallel β-sheets and two short α-helices. β-Strands A, B, part of D, E, and H form β-sheet I, and strands C, part of D, F, G, I, and J form β-sheet II. β-Sheet I contains a surface trench into which a triple helical collagen molecule fits as shown by docking

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[1] The abbreviations used are: MSCRAMM, Microbial Surface Component Recognizing Adhesive Matrix Molecules; CNA, a collagen-binding protein from *S. aureus*; ACE, a collagen-binding protein from *E. faecalis*; ELISA, enzyme-linked immunosorbent assay; GST, glutathione *S*-transferase; mAb, monoclonal antibody; PCR, polymerase chain reaction; ECM, extracellular matrix; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

TABLE I
Oligonucleotide primers used in this study

Primers	Sequence 5′ → 3′[a]
cna-(151–318) 5′	GAAGGATCC**ATAACATCTGGGAATAAATC**
cna-(151–318) 3′	GTTGTCGAC*TCA***ATTGTGCACAGTATG**
ace-(152–318) 5′	GAAGGATCCACAGCAACGGCGACTC
ace-(152–318) 3′	GTTGTCGAC*TCA*ATTTTTAACCTGTGATG
cna-(151–318)G	TTGGCCTGCTTCTGTGAT**TTTGGTTTTGTAGTTAAT**
ace-(152–318)G	**ATTAACTACAAAACCAAAA**TCACAGAAGCAGGCCAA
cna-(151–318)I	TCAAATCAAGTACGT**TGGTTTTTAAATATTAAC**
ace-(152–318) I	**GTTAATATTTAAAAACCAACGTACTTGATTTGA**
cna-(151–318)J	GTCCGTTACACTTCGACA**ATTACGAATGAACAGCAA**
ace-(152–318)J	**TTGCTGTTCATTCGTAAT**TGTCGAAGTGTAACGGAC
cna-(151–318)N	GAAGATATTTCAATT**AAGGATCAGATTCAAGGTG**
ace-(152–318)N	**TTGAATCTGATCCTT**AATTGAAATATCTTCTGTG
cna-(151–318)W	GTTTAGTTGATAATT**AGCTTGTGAATTATTAACAAAC**
ace-(152–318)W	**AATAATTCACAAGCT**AATTATCAACTAAACAATCAAG

[a] *Boldface letters* indicate sequences from *cna*-(151–318); *underlined* and *italicized letters* indicate restriction sites and stop codons, respectively, added for cloning purposes.

experiments using theoretical collagen probes of $[(Gly\text{-}Pro\text{-}Pro)_4]_3$ or $[(Gly\text{-}Pro\text{-}Hyp)_4]_3$. Site-directed mutagenesis of some residues in the putative binding trench of CNA-(151–318) abolished collagen binding (Y175K, R189A, F191A, N193K, and Y233A) or caused reduced binding affinity (N223K and N278K) (21, 22), indicating that the trench in fact constitutes the binding site. Two truncates of CNA-(151–318) that had the N-terminal 30 amino acids or the C-terminal 22 amino acids removed, respectively, were generated and were found to be unable to bind collagen CNA-(157–297) or be insoluble CNA-(181–318). CNA-(157–297) contains all residues of the trench, and CNA-(181–318) contains all but six of the residues in the binding trench, suggesting that the intact CNA-(181–318) molecule is important in presenting the trench in an active collagen binding conformation.

Recently, a collagen-binding MSCRAMM, ACE, of *E. faecalis* was identified (14). ACE has a structural organization similar to that of CNA and contains an N-terminal signal peptide, a collagen binding A region followed by the B region composed of repeated units and in the C terminus an element required for cell wall anchoring, a transmembrane domain, and a short cytoplasmic tail. The A region of ACE shares sequence similarity with the A region of CNA, and these parts of the two proteins appear to be structurally related as determined by far-UV circular dichroism analysis and computer modeling (14). These observations raised the possibility that ACE also contains a trench structure representing the collagen-binding site.

Eucaryotic cells also contain collagen-binding adhesion receptors. So far, four collagen-binding integrins have been identified on mammalian cells. These are dimeric proteins and composed of a common β chain (β_1) but different and distinct α chains (α_1, α_2, α_{10}, and α_{11}). The α chains contain a so-called inserted (I) domain that appears to be responsible for the collagen binding activity. The crystal structures of the I domains from α_1 and α_2 have been solved (23–25). In both cases these domains contained a putative ligand-binding trench that can accommodate a collagen triple helix. Thus a binding trench may be a common feature of collagen-binding proteins. In the current study, we report on a monoclonal antibody approach to examine the structure-function relationship of CNA-(151–318).

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions—Escherichia coli strain JM101 was used as a host and pQE-30 (Qiagen Inc., Chatsworth, CA) as an expression vector for cloning and expressing recombinant proteins. *S. aureus* strain Cowan I was obtained from the Istituto Sieroterapico Milanese (Milan, Italy) (26). *E. coli* strains were grown in Lennox L broth (LB) (Sigma, St. Louis, MO) or on LB agar at 37 °C overnight with antibiotics when appropriate. Staphylococci were grown in Brain Heart Infusion (Difco, Detroit, MI) broth or on Brain Heart Infusion agar at 37 °C overnight.

Generation of Monoclonal Antibodies—Monoclonal antibodies against CNA-(151–318) were produced essentially as described by Köhler and Milstein (27) with minor modifications. BALB/c mice were injected intraperitoneally five times at 1-week intervals with 50 μg of the purified recombinant protein. The antigen was emulsified with an equal volume of complete Freund's adjuvant for the first immunization, followed by three injections in incomplete adjuvant. The mice were bled, and the sera were tested for reactivity to the purified CNA-(151–318) using ELISA and Western blot. For the final immunization, the antigen was given in saline. Three days later, the lymphocytes were isolated from spleens and fused with Sp2/0 Ag.14 mouse myeloma cells at a ratio of 5:1 using 50% polyethylene glycol 4000. The suspended cells were first grown and selected in high glucose Dulbecco's modified Eagle's medium/RPMI 1640 (1:1) medium (Sigma) containing 2% hypoxantine/aminopterin/thymidine (Sigma), 2% glutamine, 2% penicillin, and 2% streptomycin. After 1 week, the hypoxantine/aminopterin/thymidine medium was progressively replaced by culturing cloned hybridomas in a serum-free medium consisting of Dulbecco's modified Eagle's medium/RPMI 1640 supplemented with 1% (v/v) Nutridoma-SR (Roche Molecular Biochemicals, Mannheim, Germany) and antibiotics. Supernatants of the cell cultures were screened by ELISA on day 10, and hybridomas positive for the antibodies against CNA-(151–318) were subcultured to a density of 1 cell per well by limiting dilution and further characterized by ELISA and Western blot. 16H9 is a mAb raised against CNA_{30-531} using the same method.

Antibody Purification and Isotyping—Supernatants of hybridoma cells were collected and centrifuged. The antibodies were purified by using ammonium sulfate precipitation of the supernatant, followed by affinity chromatography on Protein A/G-Sepharose columns according to the recommendations of the manufacturer (Amersham Pharmacia Biotech).

Isotyping of the produced monoclonal antibodies (mAbs) was performed using a Mouse-Typer sub-isotyping kit (Bio-Rad, Richmond, CA).

Routine DNA Manipulation and Transformation of E. coli—DNA preparation, purification, restriction digestion, agarose gel electrophoresis, and ligation were performed using standard methods (28) or following the manufacturer's instructions unless otherwise stated. Restriction enzymes, T4 DNA ligase, and calf intestine alkaline phosphatase were from Life Technologies (Gaithersburg, MD). DNA for sequencing was prepared using the QIAprep Spin Miniprep kit (Qiagen). Routine preparation of *E. coli* competent cells and transformation of DNA into *E. coli* were performed by a one-step procedure (29).

Cloning of ACE-(152–318)—The amino acid sequences of CNA-(30–531) and ACE-(32–367) were compared using ClustalW with the default parameters. ACE-(152–318) corresponds to CNA-(30–531) and was constructed. Primers were designed to PCR amplify the corresponding nucleotide sequence from *ace*-(32–367) (Table I) using *Pfu* DNA polymerase (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

The primers were designed such that the PCR product contained a *Bam*HI site at its 5′-end and a stop codon followed by a *Sal*I site at its 3′-end. The PCR product was digested with *Bam*HI and *Sal*I and purified. It was then ligated to *Bam*HI- and *Sal*I-digested and -dephos-

TABLE II
Combination of primers and templates used for the construction of chimeras

Chimeras	First round of PCR				Second round of PCR
	Segment 1		Segment 2		
	Template	Primers	Template	Primers	Primers
G	*cna*-(151–318)	*cna*-(151–318) 5′, *cna*-(151–318) G	*ace*-(152–318)	*ace*-(152–318)G, *ace*-(152–318) 3′	*cna*-(151–318) 5′, *ace*-(152–318) 3′
I	*ace*-(152–318)	*ace*-(152–318) 5′, *ace*-(152–318) I	Chimera G	*cna*-(151–318) I, *ace*-(152–318) 3′	*ace*-(152–318) 5′, *ace*-(152–318) 3′
J	*ace*-(152–318)	*ace*-(152–318) 5′, *ace*-(152–318) J	*cna*-(151–318)	*cna*-(151–318) J, *cna*-(151–318) 3′	*ace*-(152–318) 5′, *cna*-(151–318) 3′
L	*ace*-(152–318)	*ace*-(152–318) 5′, *ace*-(152–318) I	*cna*-(151–318)	*cna*-(151–318) I, *cna*-(151–318) 3′	*ace*-(152–318) 5′, *cna*-(151–318) 3′
N	*ace*-(152–318)	*ace*-(152–318) 5′, *ace*-(152–318) N	*cna*-(151–318)	*cna*-(151–318) N, *cna*-(151–318) 3′	*ace*-(152–318) 5′, *cna*-(151–318) 3′
W	*cna*-(151–318)	*cna*-(151–318) 5′, *cna*-(151–318) W	*ace*-(152–318)	*ace*-(152–318) W, *ace*-(152–318) 3′	*cna*-(151–318) 5′, *ace*-(152–318) 3′

phatased pQE-30 (Qiagen). The ligation mixture was transformed into *E. coli* JM101, and the cells were incubated on LB agar plates supplemented with 50 μg/ml ampicillin at 37 °C overnight to select for transformants. The construct was confirmed by restriction enzyme digestions and further verified by DNA sequencing.

Generation of Constructs Containing Chimeric Sequences between ace-(152–318) and cna-(151–318)—An overlapping PCR strategy was used for the construction. Primers that span each corresponding junction region between *ace*-(152–318) and *cna*-(151–318) were designed (Table II). PCR reactions were carried out using the proper combination of primers and templates (Table II). The construction of chimera L is described here as an example. Briefly, the primer to the 5′-end of *ace*-(152–318), *ace*-(152–318) 5′, and *ace*-(152–318)I, which spans the junction region between *ace*-(152–318) and *cna*-(151–318) were used to amplify the N-terminal quarter segment from *ace*-(152–318). The corresponding *cna*-(151–318) primers, *cna*-(151–318)I and *cna*-(151–318) 3′ were used to amplify the C-terminal three-quarters segment from *cna*-(151–318). PCR reactions were as described above for the cloning of *ace*-(152–318). After the reactions, 1 μl of a 5-fold dilution of each reaction were mixed and used as templates for the second round PCR with primers *ace*-(152–318) 5′ and *cna*-(151–318) 3′. The PCR product was purified using the Qiaquick PCR purification kit (Qiagen), digested with *Bam*HI and *Sal*I, and ligated to pQE30 vector as described above for the cloning of *ace*-(152–318). Each construct was confirmed by DNA sequencing.

Expression of Recombinant Proteins in E. coli and Protein Purification—Protein expression and purification were described previously (14, 22). Briefly, 1 liter of LB (supplemented with 50 μg/ml ampicillin) was inoculated with 40 ml of an overnight culture of a recombinant *E. coli* strain and grown at 37 °C for 3 h. Isopropyl-β-D-thiogalactoside was added, and the culture was grown for another 3 h to allow protein expression. Bacteria were harvested by centrifugation, and the cell pellets were resuspended in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) and stored at −80 °C.

The pellets were thawed and lysed in a French press. The cell debris was removed by centrifugation, and the supernatant was filtered through a 0.45-μm membrane. The supernatant was applied to a 5-ml Ni^{2+}-charged HiTrap chelating column (Amersham Pharmacia Biotech), and bound protein was eluted with a 200-ml linear gradient of 0–200 mM imidazole in 4 mM Tris-HCl, 100 mM NaCl (pH 7.9), at a flow rate of 5 ml/min. Fractions corresponding to each recombinant protein, as determined by SDS-polyacrylamide gel electrophoresis, were pooled and dialysed against HBS buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) containing 3.4 mM EDTA. Protein concentration was measured on a Beckman DU-70 UV-visible spectrophotometer at $\lambda_{280-315}$. The molar extinction coefficient of each protein was calculated using the method of Pace *et al.* (30). A single band of expected molecular weight was observed on SDS-polyacrylamide gel electrophoresis after Coomassie Blue staining for the different chimeras.

Enzyme-linked Immunosorbent Assay—Microtiter wells were coated overnight at 4 °C with 100 μl of a 10 μg/ml concentration of each recombinant protein in 50 mM sodium carbonate, pH 9.5. To block additional protein-binding sites, the wells were treated for 1 h at 22 °C with 200 μl of PBS containing 2% (w/v) bovine serum albumin (BSA) and then washed five times with PBST (PBS with 0.1% v/v Tween 20). Indicated amounts of each mAb dissolved in 100 μl of PBS with 2% BSA were added to the wells and incubated for 2 h at 22 °C. Plates were then extensively washed with PBST and incubated for 1 h with a rabbit anti-mouse IgG conjugated to horseradish peroxidase (1:500 dilution; Dako, Gostrup, Denmark). After washing, binding was quantitated using the substrate *o*-phenylenediamine dihydrochloride (Sigma) and measuring the absorbance at 492 nm in a microplate reader (Bio-Rad). The concentration required for half-maximal binding of the antibody to CNA-(151–318) was used to calculate the apparent K_D of each mAb.

Iodination of Collagen—Type II collagen was prepared from bovine nasal septum as described by Strawich and Nimni (31). Carrier-free ^{125}I (15 mCi/μg) was from Amersham Pharmacia Biotech. Collagen was labeled using the iodogen coated-tube technique as recommended by the manufacturer (Pierce, Rockford, Ill). The specific activity of the radiolabeled ligand was estimated to be 4×10^6 cpm/μg.

Inhibition of the Collagen Binding of CNA-(151–318) by the mAbs—Microtiter wells were coated with CNA-(151–318) as in ELISA described above and then incubated with ^{125}I-collagen (8×10^4 cpm) in the presence of indicated amounts of each mAb for 1 h. After extensive washing, the radioactivity in each well was measured in a γ-counter. The amount of radioactivity in each well was compared with that bound to the MSCRAMM in the absence of mAb, which was set to 100%. All experiments were done in duplicate. The concentration of each mAb required to inhibit half the binding of collagen to CNA-(151–318) (IC_{50}) was calculated.

Binding of ^{125}I-collagen to S. aureus Cells—Binding of ^{125}I-labeled collagen to *S. aureus* cells was quantitated as described previously (32). Briefly, 5×10^8 *S. aureus* Cowan I cells were incubated with 5×10^4 cpm of ^{125}I-collagen in 0.5 ml of PBS containing 0.1% BSA and 0.1% Tween 80. The mixture was rotated in an end-over-end mixer for 1 h at 22 °C. The reaction was stopped by the addition of 2.5 ml of ice-cold PBS containing 0.1% Tween 80, and the tubes were centrifuged at $1400 \times g$ for 10 min. After aspiration of the supernatant, the radioactivity associated with the cell pellet was determined in a γ-counter. Radioactivity recovered in tubes incubated in the absence of bacteria (400–600 cpm) was considered background and was subtracted from bacteria-containing samples. Duplicate samples were analyzed.

To analyze the inhibitory effect of the mAbs on the binding to soluble collagen, bacteria were preincubated with indicated amounts of mAbs for 1 h before the addition of ^{125}I-collagen. The binding assay was carried out as described above.

To examine the displacing effect of the mAbs on collagen binding, bacteria were preincubated with ^{125}I-collagen for 1 h. After centrifugation, the supernatant was removed, the bacteria-collagen pellet was washed and resuspended in PBS containing 0.1% BSA and 0.1% Tween 80 with indicated amounts of mAb, and incubated for another hour. The amount of residual collagen bound to bacteria was determined as above.

Adherence of S. aureus Cells to Collagen Substrates—Microtiter wells were incubated with 100 μl of 10 μg/ml type II collagen in 50 mM sodium carbonate, pH 9.5, overnight at 4 °C. Remaining protein-binding sites in

TABLE III
Summary of the immunoglobulin isotypes, the apparent K_D values of the mAbs, and the concentration required to inhibit 50% of maximum binding of type II collagen to CNA-(151–318)

Antibody	Isotype	K_D	IC_{50}
		M	
1F6	IgG_{2b}-κ	4.5×10^{-9}	3.7×10^{-8}
1H1	IgG_1-κ	1.9×10^{-9}	1.3×10^{-8}
2B1	IgG_1-κ	0.65×10^{-9}	1.1×10^{-8}
2B3	IgG_{2a}-κ	1.3×10^{-9}	1.0×10^{-8}
3B12	IgG_1-κ	4×10^{-9}	8.6×10^{-8}
3D3	IgG_{2a}-κ	1.26×10^{-9}	4.2×10^{-8}
5D12	IgG_1-κ	5.8×10^{-9}	1.0×10^{-8}
5G4	IgG_1-κ	4.2×10^{-8}	1.6×10^{-8}
5H1	IgG_1-κ	8.6×10^{-9}	1.3×10^{-8}
7C2	IgG_{2a}-κ	1.6×10^{-9}	1.1×10^{-8}
7E2	IgG_1-κ	1.6×10^{-9}	1.2×10^{-8}
7G2	IgG_1-κ	1.15×10^{-9}	1.1×10^{-8}
8E6	IgG_1-κ	1.0×10^{-9}	1.1×10^{-8}
8H10	IgG_{2a}-κ	1.14×10^{-9}	1.1×10^{-8}
9A4	IgG_1-κ	1.15×10^{-9}	1.5×10^{-8}
9F11	IgG_1-κ	1.28×10^{-9}	6.3×10^{-9}
9G3	IgG_1-κ	1.02×10^{-9}	8.1×10^{-9}
9G7	IgG_1-κ	1.03×10^{-9}	4.1×10^{-9}
10G5	IgG_1-κ	1.6×10^{-9}	4.4×10^{-9}
11D5	IgG_{2a}-κ	2.27×10^{-9}	4.0×10^{-9}
11H11	IgG_{2a}-κ	2.5×10^{-9}	1.7×10^{-8}
12H10	IgG_1-κ	1.46×10^{-9}	6.1×10^{-9}
16H9	IgG_1-κ	5×10^{-8}	

the wells were blocked by incubating with 200 μl of 2% BSA for 1 h at 22 °C. After washing with PBST, the wells were incubated with 100 μl of a suspension of Cowan I (1×10^7 cells/ml) at 22 °C for 2 h, and then washed extensively with PBST. Adherent cells were detected by incubation of the wells for 1 h with 100 μl of a horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:500 dilution), which presumably bound to protein A on the staphylococcal cells. Color development and measurement was as described above.

To study the inhibition of bacterial adherence to collagen by the mAbs, cells were preincubated for 30 min with the indicated amounts of antibody. The cells were then transferred to the collagen-coated wells, incubated for 2 h and processed as above.

In displacement experiments, bacteria were first incubated in collagen-coated wells for 1 h, and adherent bacteria were then incubated with increasing concentrations of the indicated mAbs for 2 h. Bacteria that remained attached to collagen were detected by the addition of horseradish peroxidase-conjugated rabbit anti-mouse IgG as described above. As controls for the adherence assay, the adhering bacteria were also detected in selected experiments using crystal violet staining as described previously (33). The results of the two detection methods were essentially identical.

RESULTS

Initial Characterization of Monoclonal Antibodies—In this study we have analyzed 22 monoclonal antibodies raised against recombinant CNA-(151–318) and, mAbs 7E8 and 16H9, raised against the recombinant full-length A region, CNA-(30–529). The isotypes and apparent K_D (the concentration required for half maximum binding) were determined by ELISAs (Table III). The majority of the mAbs were IgG_1-κ, six were IgG_{2a}-κ (2B3, 3D3, 7C2, 8H10, 11D5, and 11H11), and one (1F6) was an IgG_{2b}-κ. The majority of the mAbs exhibited similar apparent K_D values in the 10^{-9} M range, for binding to CNA-(151–318) adsorbed in microtiter wells. 5G4 and 16H9 showed a slightly lower affinity with apparent K_D values of 4.2×10^{-8} M and 5×10^{-8} M, respectively.

Initial screening of the different mAbs showed that 7E8 reacted with the N-terminal fusion component containing the six His residues found in a number of recombinant proteins expressed using the pQE30 vector.

Mapping of Epitopes Recognized by mAbs—In attempts to map the epitopes recognized by the different mAbs, a set of overlapping synthetic peptides were made. Each of the peptides was 25 amino acids long, and the set spanned the entire CNA-(151–318) sequence. However, none of the mAbs bound any of the peptides in ELISA assays (data not shown). Nor did any of the peptides in solution interfere with the binding of the mAbs to adsorbed CNA. A set of GST fusion constructs, each of which encoded a stretch of 50 to 55 amino acids of CNA-(151–318) sequence, were constructed and examined in an ELISA. However, none of the mAbs reacted with these fusion peptides (data not shown). Taken together, these results suggest that all mAbs reacted with conformationally dependent epitopes that were not found in the synthetic peptides nor in the GST fusion proteins. Thus epitope mapping required an alternative strategy. We have previously shown that *E. faecalis* can express a collagen-binding MSCRAMM, ACE, that has a structural organization similar to that of CNA (14). The A region (the binding domain) of ACE was predominantly composed of β-strands as determined by its far-UV circular dichroism spectrum. When the region in ACE corresponding to CNA-(151–318) was analyzed using Swiss Model, the predicted structure had a backbone folding highly similar to that of CNA-(151–318) determined by x-ray analysis of the protein crystal. The corresponding region in ACE was then designated ACE-(152–318). Fig. 1*A* showed the result of ClustalW alignment of the amino acid sequences of CNA-(151–318) and ACE-(152–318) using the default parameters. The two sequences had 29% overall identity and 40% overall similarity. Higher homology was observed in regions corresponding to CNA-(151–318) β-strands A, B, and H and their flanking residues, which form a major part of the putative collagen-binding trench on one surface of the molecule. ACE-(152–318) was cloned, and the purified protein was tested for its reactivity with the anti-CNA mAbs. Except for 7E8, which binds the His-tag, the anti-CNA mAbs did not recognize ACE-(152–318) (Table IV). Thus, ACE-(152–318) could be used as an inert template for constructing chimeric proteins for epitope mapping where regions of CNA-(151–318) replaced the corresponding sequences from ACE-(152–318).

A large number of chimeric proteins were constructed. However, some of these proteins had a low degree of solubility when expressed and/or were not recognized by the mAbs, suggesting that the structures of these proteins were significantly different from that of CNA-(151–318). The chimeric proteins useful for epitope mapping are presented in Fig. 1*B*, and the reactivity of the different mAbs with the different chimeric proteins is summarized in Table IV. A summary of the epitope mapping is shown in Fig. 1*C*.

Effects of mAbs on Binding of ^{125}I-collagen to CNA—The effects of the different mAbs on the binding of ^{125}I-collagen to CNA-(151–318) adsorbed in microtiter wells were examined. The mAbs raised against CNA-(151–318) interfered with the MSCRAMM ligand interaction when added to the microtiter wells at 2 μg/well (Fig. 2). Most of these mAbs had drastic effects and inhibited collagen binding of CNA-(151–318) by at least 80%. mAb 7E2 was less potent and reduced binding by 57%, compared with the ^{125}I-collagen binding observed in the absence of mAbs. Mab 7E8, which reacted with the His-tag, and mAb 16H9 had no effect on ^{125}I-collagen binding to the adsorbed CNA-(151–318). The concentration-dependent inhibition of several representative mAbs on the binding of ^{125}I-collagen to CNA-(151–318) is shown in Fig. 3. The mAbs that do affect binding do so in a concentration-dependent manner. The concentration of each blocking mAb required to inhibit 50% of maximum binding (IC_{50}) was determined (Table III). The majority of the mAbs exhibited similar IC_{50} values between 0.4 and 1.7×10^{-8} M. mAb 1F6 required slightly higher concentrations with an IC_{50} of 3.7×10^{-8} M. Some mAbs, *e.g.* 11H11 and 8E6, appeared to inhibit ^{125}I-collagen binding to CNA-

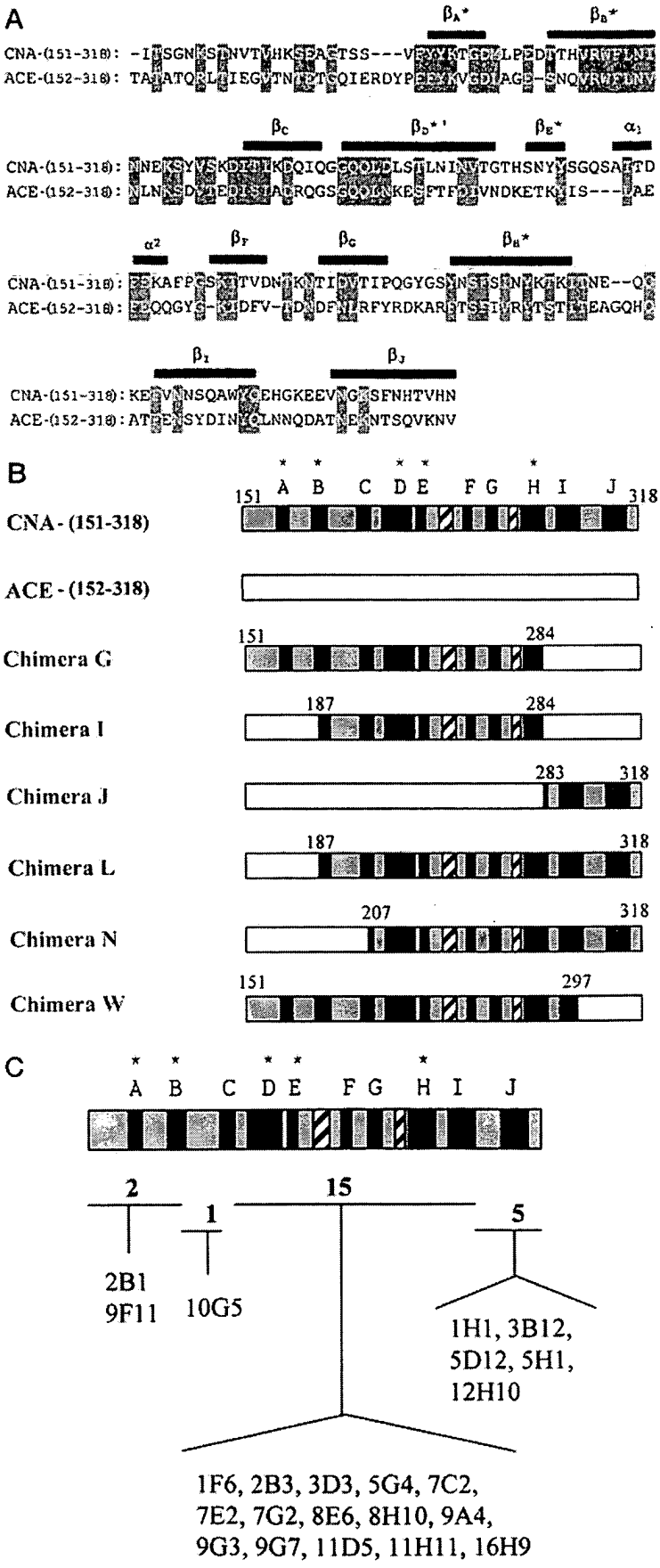

FIG. 1. *A*, sequence alignment of CNA-(151–318) and ACE-(152–318). ClustalW with default parameters was used. *Shaded* amino acid residues are identical and similar residues. The β-strands and α-helices of CNA-(151–318) determined by x-ray crystallography are indicated by *dark bars* above the corresponding regions of CNA-(151–318). β-strands A, B, a part of D, E, and H are the ones that form the observed trench and are indicated by *asterisks*. *B*, schematic illustration of the chimeras. CNA-(151–318) sequences are represented by *shaded bars*, while ACE-(152–318) are represented by *blank bars*. *Dark-shaded boxes* represent β-strands, and *striped boxes* α-helices of CNA-(151–318). The *asterisks* indicate the β-strands that form the putative ligand binding trench. The *numbers* indicate the positions of the amino acid residues in CNA-(151–318). *C*, summary of the localization of CNA-(151–318) epitopes recognized by the mAbs. *Horizontal lines* beneath CNA-(151–318) represent regions where various mAbs were mapped. The *numbers* above each line indicate the number of mAbs in the region (includes 16H9, but does not include the anti-His-tag mAb 7E8).

TABLE IV
Reactivity of the mAbs against CNA-(151–318), ACE-(152–318), and the chimeras
The reactivities were determined using ELISAs.

mAb	CNA-(151–318)	ACE-(152–318)	G	I	J	L	N	W
7E8[a]	+	+	+	+	+	+	+	+
1F6	+	–	+	+	+	+	+	+
1H1	+	–	–	–	+	+	–	–
2B1	+	–	+	–	–	–	–	+
2B3	+	–	+	+	–	+	+	+
3B12	+	–	–	–	+	+	–	–
3D3	+	–	+	+	–	+	+	+
5D12	+	–	–	–	+	+	–	–
5G4	+	–	+	+	–	+	(+)[b]	+
5H1	+	–	–	–	+	+	–	–
7C2	+	–	+	+	–	+	+	+
7E2	+	–	+	+	–	+	+	+
7G2	+	–	+	+	–	+	+	+
8E6	+	–	+	(+)	–	+	+	+
8H10	+	–	+	+	–	+	+	+
9A4	+	–	+	+	–	+	+	+
9F11	+	–	+	–	–	–	–	+
9G3	+	–	+	–	–	+	(+)	+
9G7	+	–	+	+	–	+	+	+
10G5	+	–	+	(+)	–	(+)	–	+
11D5	+	–	+	+	–	+	+	+
11H11	+	–	+	+	–	+	+	+
12H10	+	–	+	+	–	–		
16H9	+	–	+	+	–	+	(+)	+
Total reacting mAbs[c]	24	1	19	16	7	22	16	19

[a] 7E8 is an anti-His tag monoclonal antibody.
[b] Parentheses indicate weak reactions in ELISAs.
c The number includes positive reactions with 7E8.

(151–318) partially and allowed 20% of maximum binding even at high concentrations of mAbs. It is noteworthy that the relative potency of the different mAbs are similar using the inhibition and the direct ELISA methods.

Effects of mAbs on the binding of ^{125}I-collagen to S. aureus Cells—We also examined the effects of mAbs on the binding of soluble ^{125}I-collagen to *S. aureus* cells. Most of the mAbs examined substantially inhibited collagen binding to bacteria when added at 1 μg per 0.5 ml, and greater than 80% inhibition was achieved at 5 μg per 0.5 ml (Fig. 4A). The control mAbs 16H9 and 7E8 had no effect on collagen binding to bacteria. The tendency of concentration-dependent effects on collagen binding to bacteria by inhibiting antibodies shown in Fig. 4*A* was further examined and firmly established for some selected mAbs, *e.g.* 9G7, 11H11, 8E6, and 16H9 (Fig. 5*A*).

mAbs Can Displace ^{125}I-collagen Bound to S. aureus Cells—Experiments were performed where ^{125}I-collagen was incubated with *S. aureus* cells for 1 h to allow the radiolabeled ligand to bind to bacteria. The cells were then washed and incubated for another hour in the presence of the different mAbs to explore the possibility that the mAbs can displace the cell-bound ^{125}I-collagen. With the exception of the control mAbs 7E8 and 16H9, all mAbs examined caused some displacement of cell-bound ^{125}I-collagen (Fig. 4*B*). However, the displacement potency of the different mAbs varied substantially. Some mAbs such as 5G4, 8E6, 10G5, 11D5, and 11H11 only caused partial release of cell-bound ^{125}I-collagen, whereas other mAbs such as 3B12, 5H1, 7G2, 9A4, and 9G7 displaced all cell-bound ^{125}I-collagen. In general, an ineffective inhibitor of collagen binding to bacteria was also a poor displacer of cell-bound ^{125}I-collagen (*e.g.* mAbs 8E6 and 10G5). However, some good inhibitors were poor displacers (*e.g.* 5G4, 7C2, 8H10, and 11H11). Furthermore, there was no correlation between apparent affinity for CNA-(151–318) and displacing potential. For example, the good displacers 1F6, 3B12, and 5H1 had higher apparent K_D values than did the poor displacers 3D3, 8E6, and 8H10.

The concentration-dependent displacement of cell-bound ^{125}I-collagen was examined for some selected mAbs (Fig. 5*B*). All displacing mAbs examined showed a concentration-dependent activity. However, whereas the good displacer 9G7 caused total release of cell-bound ^{125}I-collagen at 0.5 μg/ml, the poor displacer 11H11 only released a maximum of 50% of cell-bound ^{125}I-collagen even when the antibody was added at 2.5 μg/ml.

Effects of mAbs on the Adherence of Bacteria to a Collagen Substrate—Selected mAbs were examined for the ability to inhibit bacterial adherence to a collagen substrate (Fig. 6*A*). Of the mAbs analyzed, those that inhibited the binding of ^{125}I-collagen to staphylococci also interfered with the attachment of bacteria to a collagen substrate. Furthermore, mAbs 9G7 and 11H11, which were good inhibitors of ^{125}I-collagen binding to bacteria, also effectively inhibited the attachment of staphylococci to the collagen substrate. On the other hand, mAb 8E6 was a less potent inhibitor of bacteria's binding of soluble collagen and attachment to collagen substrate, although total inhibition could be achieved provided that sufficient amounts of 8E6 were added. mAb 16H9, which did not inhibit binding of ^{125}I-collagen to bacteria, also did not affect attachment of cells to collagen.

mAbs Can Displace Adherent Bacteria from a Collagen Substrate—Remarkably, some mAbs caused the detachment of staphylococcal cells that had adhered to the collagen substrate during a 1-h incubation before the addition of mAbs (Fig. 6*B*). mAb 9G7, which effectively released ^{125}I-collagen bound to the bacterial surface, also effectively detached pre-attached bacteria from a collagen substrate. mAb 11H11, which was an ineffective displacer, only caused partial detachment, whereas 8E6 and 16H9, which were essentially inactive in the detachment assay, also had little or no effect, respectively, in the displacement assay. Although the number of mAbs that were analyzed in all assays is limited, our data suggest a direct correlation between the ability to displace bacteria bound ^{125}I-collagen and detach pre-attached bacteria from a collagen substrate.

DISCUSSION

S. aureus is the predominant bacterium associated with septic arthritis, and CNA, the collagen-binding MSCRAMM of *S. aureus*, is a virulence factor in experimental septic arthritis (2). In addition, a recombinant fragment of CNA (CNA-(30–529)) was effective in protecting mice against *S. aureus* induced septic death (3), raising the possibility of using CNA as a vaccine to prevent staphylococcal infections. These observations prompted us to further analyze CNA using a monoclonal antibody approach.

A panel of 22 mAbs were raised against CNA-(151–318) and characterized. None of these mAbs were reactive with synthetic peptides or recombinant peptides containing 25 to 55 residues that span the entire length of CNA-(151–318), suggesting that the mAbs recognized conformationally dependent epitopes. To locate the epitopes recognized by the different mAbs, an alternative approach was necessary. Because none of the anti-CNA-(151–318) mAbs recognized the corresponding segment of the collagen-binding MSCRAMM of *E. faecalis*, ACE, we could use a chimeric protein strategy where segments of CNA-(151–318) were grafted on ACE-(152–318). Chimeras G, I, J, N, and W were recognized by the different mAbs, suggesting that these chimeras were able to properly present the epitopes for antibody recognition. The number of mAbs reacting with the individual chimeras was approximately proportional to the length of the CNA-(151–318) sequence in each chimera, indicating that mAbs recognized epitopes located throughout the CNA-(151–318) molecule. In all, two mAbs were mapped to the N-terminal quarter, 15 (including 16H9) mapped to the central region, and 5 mapped to the C-terminal end of CNA-(151–318).

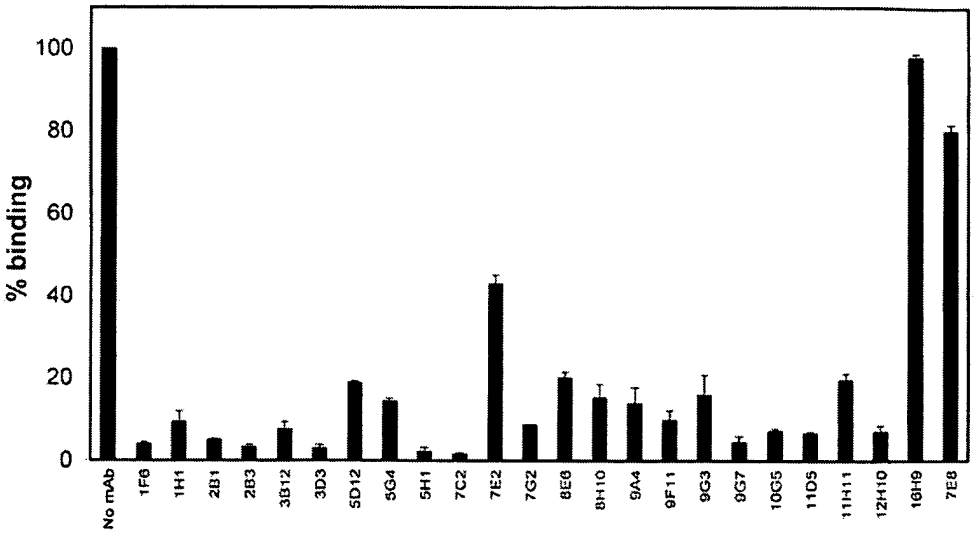

FIG. 2. **Effect of different mAbs on the binding of ^{125}I-collagen to immobilized CNA-(151–318).** Recombinant CNA-(151–318) protein was immobilized onto microtiter wells (1 μg in 100 μl) and probed with ^{125}I-collagen (8 × 10^4 cpm) in the presence of 2 μg of each mAb. After washing with PBS containing 0.1% Tween 80, binding of the ligand was quantitated by counting the wells in a γ counter. The binding of ^{125}I-collagen to CNA-(151–318) in the absence of antibody was set as 100% binding. *Bars* represent means ± S.D. with duplicate testing.

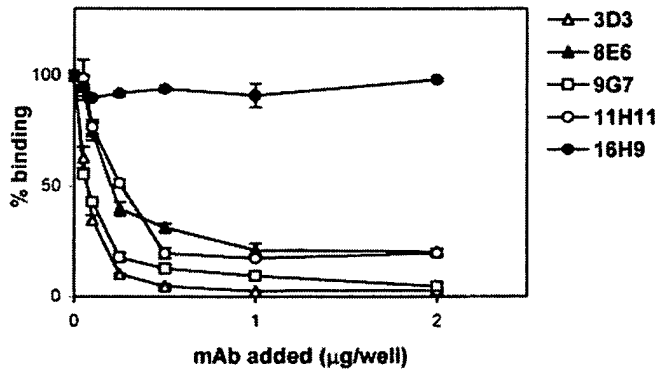

FIG. 3. **Concentration-dependent effect on ^{125}I-collagen binding to immobilized CNA-(151–318) by the different mAbs.** Microtiter wells were coated with CNA-(151–318) protein (1 μg in 100 μl) and incubated with ^{125}I-collagen (8 × 10^4 cpm) in the presence of increasing amounts of indicated mAbs. Collagen binding was quantitated as described in Fig. 2. The binding of ^{125}I-collagen to immobilized CNA-(151–318) in the absence of antibody was set as 100% binding. Data are reported as means ± S.D.

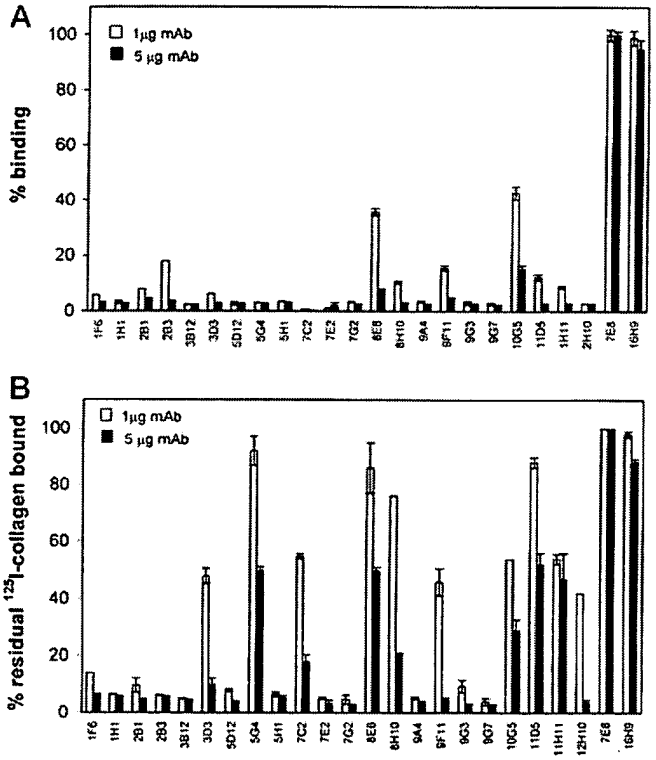

FIG. 4. *A*, different mAbs inhibit binding of ^{125}I-collagen to *S. aureus* cells. *S. aureus* Cowan I cells (5 × 10^8 cells) were preincubated with indicated amounts of mAbs prior to the addition of ^{125}I-collagen (5 × 10^4 cpm). Collagen binding was quantitated as described under "Experimental Procedures." Results are expressed as percentage of the ligand binding in the absence of mAb. *Error bars* indicate standard deviations from the mean of duplicate data points. *B*, different mAbs displace ^{125}I-collagen from *S.aureus* cell surface. *S. aureus* Cowan I cells (5 × 10^8) were preincubated with ^{125}I-collagen (5 × 10^4 cpm) for 1 h. The cells were then harvested by centrifugation and incubated with indicated amounts of mAbs for another 1 h. The amount of the residual collagen bound to bacteria was determined in a γ counter. Duplicate samples were used for each data point.

We were not able to map the epitopes to a finer resolution, because manipulations in the central region of CNA-(151–318) resulted in insoluble proteins.

Most of the anti-CNA-(151–318) mAbs in this study were able to effectively inhibit the binding of soluble collagen to CNA-(151–318) and *S. aureus* cells, as well as interfere with the attachment of bacteria to collagen substrate. No significant differences were observed between interactions with soluble or immobilized collagen in these assays.

The ability of some mAbs to displace collagen from the MSCRAMM and in effect detach *S. aureus* cells adhering to collagen substrate is perhaps the most remarkable observation of this study. Here we report that bacteria allowed to adhere to a collagen substrate can subsequently be displaced by mAbs. We have in preliminary experiments shown that the bacterial adhesion period could continue for up to at least 5 h before the mAbs were added without loss of displacing activity (data not shown).

The results of these studies allowed us to make several conclusions. First, the epitopes recognized by CNA mAbs are distributed throughout the entire structure of CNA-(151–318) rather than being located to one subdomain. Second, mAbs with good inhibiting and displacing activities (*e.g.* 2B1, 9A4, 9G7, and 3B12) are located throughout the structure of CNA-

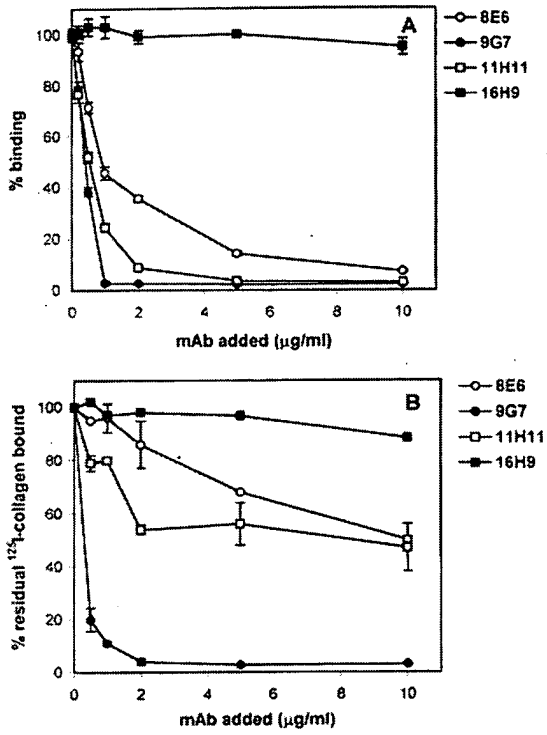

FIG. 5. **Dose-dependent effects on the association and dissociation of ^{125}I-collagen from *S. aureus* by different mAbs.** *A*, inhibition. *S. aureus* Cowan I cells (5×10^8 cells) were preincubated with increasing concentrations of mAbs 9G7, 11H11, 8E6, and 16H9 before the addition of ^{125}I-collagen. Cell-bound collagen was determined in a γ counter. Data are expressed as the percentage of ligand binding to bacteria in the absence of mAbs and represent the mean ± S.D. of duplicate measurements. *B*, displacement. *S. aureus* cells were preincubated with ^{125}I-collagen as reported in Fig. 4*B*. Increasing amounts of indicated mAbs were then added to the bacteria-collagen complexes and incubated for 1 h. Residual ^{125}I-collagen associated with bacteria was quantitated in a γ counter. *Bars* represent standard deviations from the means of duplicate measurements.

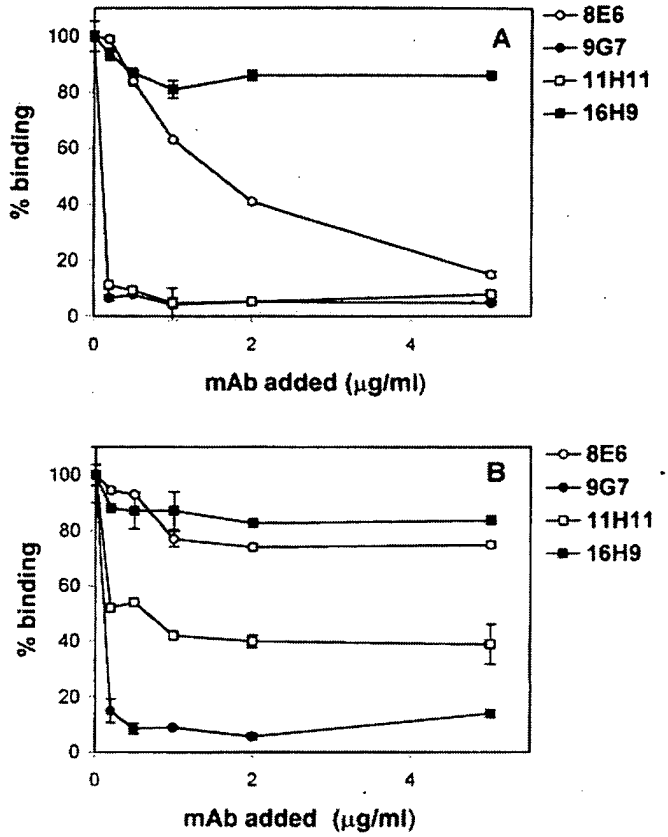

FIG. 6. **Dose-dependent effects on the attachment and detachment of *S. aureus* cells from collagen substrate by different mAbs.** *A*, inhibition. Microtiter wells were coated with collagen type II as described under "Experimental Procedures." Bacteria (1×10^6 cells/well) were preincubated with increasing amounts of indicated mAbs before being added to the wells. Adherent cells were detected by incubation with peroxidase-conjugated rabbit anti-mouse IgG. *B*, displacement. Cells were allowed to adhere to collagen-coated plates. After washing, indicated amounts of mAbs were added to the wells and incubated for 2 h. The number of residual attached bacteria after washing was quantitated by using peroxidase-conjugated rabbit anti-mouse IgG. Data are expressed as percentage of attached bacteria in the absence of mAbs. The values represent means ± S.D. of duplicate measurements.

(151–318). Considering the rope-like structure of the collagen molecule, it is unlikely that the entire CNA-(151–318) on ligand binding makes direct contact with collagen. Some inhibiting mAbs may interact with residues that directly contact collagen and act as competitive inhibitors, whereas other mAbs probably bind to the MSCRAMM outside the ligand binding site and for sterical reasons interfere with the interactions between CNA and collagen. Third, the displacing mAbs were effective in inhibiting collagen binding, however, not all the inhibiting antibodies were good displacing mAbs. Fourth, mAbs that are effective inhibitors but poor displacers such as 5G4, 8H10, 11D5, and 11H11 recognize epitopes located in the central segment of CNA-(151–318). These mAbs could potentially inhibit binding by directly interfering with the action of the putative ligand binding trench. While in collagen-CNA complex, the binding trench is protected by the collagen molecule, therefore the mAbs exhibited poor displacing abilities. Fifth, it seems that the displacement ability of some of the mAbs does not correlate with their apparent affinity for CNA-(151–318), or their ability to inhibit CNA-(151–318) binding to collagen. Sixth, some of the effective displacers, 1H1, 3B12, 5D12, and 5H1, recognize chimera J and appear to react with epitopes located on the backside of the CNA-(151–318) structure. Is it possible that these mAbs work by causing conformational changes in CNA-(151–318), resulting in conformation(s) with low affinity for collagen?

The concept of collagen receptor occurring in two conformational states is not unique to CNA. The I domain of integrin α_2 (α_2I) crystallized in the presence and absence of a collagen triple helical peptide indicated two conformational states for α_2I, an "active" state which was observed in complex of the collagen peptide and an "inactive" state observed in the absence of collagen (34). Mutagenesis studies on α_2I revealed that some residues that affected collagen binding were at the α-helices far away from the ligand-binding site. The authors proposed that these residues were important for maintaining the active conformation of the molecule (35). Further work is needed to reveal the mechanism of action of the anti-CNA-(151–318) mAbs that can cause ligand displacement. Such studies are underway.

The finding that some mAbs are able to detach staphylococci from surfaces coated with collagen raises the possibility that these mAbs may be used as therapeutic agents. In general, antibodies to infective agents generated through active vaccination or administered in passive immunization are used in prophylaxis to prevent the microbe from causing an infection. Antibodies capable of detaching bacteria colonization in host tissue, however, could potentially be used after an infection has been established. This strategy could be important in infections such as bacterial arthritis and osteomyelitis where *S. aureus* are notoriously difficult to be eradicated by the use of antibiotics alone. A detaching antibody would potentially force the

microbe into suspension where it would be more susceptible to the host phagocytes and to attacks by antibiotics. Thus a therapeutic approach involving a combination of antibiotics and detaching antibodies may be worth exploring.

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